I. CHROMATOGRAPHIC SEPARATION OF ORGANIC ACIDS

II. ORGANIC ACIDS OF SORGHUM SIRUP AND JUICE

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By

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I. CHROMATOGRAPHIC SEPARATION OF ORGANIC ACIDS

II. ORGANIC ACIDS OF SORGHUM SIRUP AND JUICE

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INTRODUCTION

Since previous investigations of the organic acids of sorghum sirup and juice have been qualitative this investigation was undertaken to determine these acids quantitatively.

Chromatographic procedures for quantitative separation and determination of organic acids have recently been developed. Because of its automatic features, the progressively changing chromatographic solvent system was chosen to be adapted to the separation of the acids of sorghum sirup and juice. The development of a suitable system afforded an opportunity for theoretical study of the chromatographic technique.

The objectives of the sorghum acid study were (1) to investigate the acids of sorghum sirups made from some common varieties of sorghum juice; and (2) to investigate the seasonal variation of the acids of sorghum juice.

CHROMATOGRAPHIC SEPARATION OF ORGANIC ACIDS

REVIEW OF LITERATURE

Attempts at quantitative chromatographic separation of organic acids were unsuccessful until partition chromatography was applied. Partition chromatography was developed by Martin and Synge (13) as an extension of an attempt to produce an efficient countercurrent contacting machine. Their method was originally developed for separating mixtures of acetylated amino acids. They used an aqueous phase held immobilized on silicic acid and a chloroform-1-butanol mobile phase.

Isherwood (8) replaced the aqueous phase of Martin and Synge with 0.5-normal sulfuric acid and achieved quantitative separation of certain organic acids using stepwise change of chloroform-butanol concentration. The 0.5-normal sulfuric acid was used to depress the ionization of acids such as oxalic and fumaric which would otherwise give wide bands. The occurrence of the wide bands was explained by the rapid increase in the partition coefficient with dilution.

The method of Isherwood was applied to lactic and succinic acids in foods by Clabron and Patterson (6); to fumaric acid in animal tissues by Marshall, Orten and Smith (12); and to various combinations of thirty-six acids by Marvel and Rands (14).

Bulen, Varner and Burrell (4) reported separation of 16 biologically important organic acids after much rechromatographing. Phares, Mosbach, Denison and Carson (16) separated the acids which could not be separated with chloroform-butanol by rechromatographing with ethyl ether. They used Celite instead of silicic acid with no apparent difference and hence suggested that the silicic acid was not active in the separation process.

Donaldson, Tulane and Marshall (7) used an apparatus which gradually and automatically increased the concentration of butanol in chloroform used as eluent. They determined the butanol concentration by means of density measurements of eluent. The measurement of the butanol concentration of the eluent was subject to errors of evaporation and required repetition of density measurements of eluent each time the butanol concentration was changed.

EXPERIMENTAL APPARATUS AND PROCEDURE

The apparatus used was the closed system shown in Figure 1. The long mixing chamber (M) and the tube with a small orifice connecting it to the upper chamber (U) were used for progressive changing of the solvent. The solvents used were C.P. chloroform washed with water and redistilled, and C.P. butanol redistilled over potassium carbonate. The mixing chamber was filled with chloroform, connected by means of the side arm to a pressure unit (9), brought to the desired pressure, and sealed by means of the stopcock on the side arm. The upper chamber was filled with butanol in chloroform of the varying concentrations to be discussed later. This chamber was then connected to the constant-pressure device of Mader and Mader (9) at the same pressure as already present in the mixing chamber. The connecting stopcock was then opened slightly and the mixing was automatic thereafter. The collection mechanism used was that of Mader and Mader (10). Fractionation of the eluent was accomplished by the use of the capacity siphon pipet of Mader and Mader (11) shown in Figure 2.

The column was prepared with the various weights of silicic acid (Mallinckrodt's for chromatographic analysis) to be listed. In all experiments 0.7 ml. of 0.5-normal sulfuric acid per gram of silicic acid was used. This

mixture was slurried with chloroform and compressed into a compact column. Two grams of pure silicic acid were then slurried on top of this column and pressed to a compact layer. A 2-ml. aliquot of prepared sorghum juice or sirup was forced into the upper layer of the column. After a few ml. of chloroform were placed on top of this prepared column it was ready for development.

The collected 5-ml. fractions were titrated by means of a microburette with 0.01 normal sodium hydroxide to the phenol red end point.

PEAK VOLUME-CONCENTRATION*

Determination of the butanol concentration of the eluent may be accomplished by means of a generalized volume-concentration equation that was developed as follows:

If:

A = Volume decimal fraction of butanol in chloroform in the upper chamber.
Y = Volume decimal fraction of butanol in chloroform passing into column.
X = Volume of eluent in ml.
B = Volume of mixing chamber in ml.

then,

$$B(Y + dY) = BY - YdX + AdX$$

which by integrating from X=0 to X, and Y = 0 to Y gives

$$X = B \ln \frac{A}{A - Y} .$$

This equation permits the calculation of the concentration of the eluent with any desired values of A and B. These relationships may be represented by plotting log (A - Y)

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* The material presented in this section has been accepted for publication in Analytical Chemistry.

against X for a particular pair of values for A and B as shown in Figure 4 where A is 0.5 and B is 250.

This equation was checked by determining the refractive index of the liquid in the lower chamber for various values of A and X and using a standard curve of refractive index against concentration. Concentrations Y as predicted by the equation and as determined by the refractive index were always within 0.5% of each other. Use of this equation requires a mixing chamber similar to the one described that will insure complete mixing, and a system of measuring the volume of eluent without evaporation.

Recently equations have been described by Alm, Williams and Tiselius (1) and Cherkin, Martinez and Dunn (5) which after suitable mathematical manipulation are identical with the one described above.

Chromatograms of sorghum juice and sirup were run using columns of 1-cm. diameter weighing from 6 to 12 grams, and A values of 0.35, 0.50, 0.60, and 0.70. The peak volume-concentrations of the acids were not changed by these various column weights except where separation did not occur, as for citric and tartaric acids on a 6-gram column.

A plot of the peak volume-concentrations of the five major acids of sorghum for the various A values, keeping B constant at 250, is shown in Figure 3. This graph shows that the peak volume-concentration for each acid may be expressed by the general equation $\log(A - Y) = mX + b$.

The values of m and b were determined from the experimental points by the method of least squares.

The peak volume-concentration equations for the five acids are:

Aconitic	$\log (A - Y) = -.00515X + .268$
Oxalic	$\log (A - Y) = -.00403X + .225$
Malic	$\log (A - Y) = -.00308X + .079$
Citric	$\log (A - Y) = -.00294X + .162$
Tartaric	$\log (A - Y) = -.00270X + .153$

Each equation is characteristic of the particular acid and is useful in identification of the acid.

The following opposed factors influence the choice of a solvent system for a particular separation:

1. Peaks are higher (a) the shorter the column; (b) the less the eluent volume required.

2. Separation is better (a) the longer the column; (b) the larger the eluent volume.

The desired system is the one which will separate the acids and give the highest peaks.

Regarding the column size, a 10-gram 1-cm. column will separate all the sorghum acids. Since citric and tartaric acids do not separate on a 6-gram column and do on a 10-gram column, shorter columns are preferable only if the acids to be separated do not include citric and tartaric.

A graph such as that of Figure 3 may be used to determine the least volume at which good separation will occur. The A = 0.70 system, although it requires the least volume, does not separate malic and oxalic acids because their peak volume-concentrations are too close to each other to allow satisfactory fractionation. The A = 0.35 system gives the

best separation but peaks are flat and spread out. This makes it difficult to determine where elution of the acid began and increases the blank error.

As the graph indicates, the A = 0.60 system provides separation with the least volume; therefore this system and the 10-gram column were chosen for separation of the acids of sorghum sirup and juice.

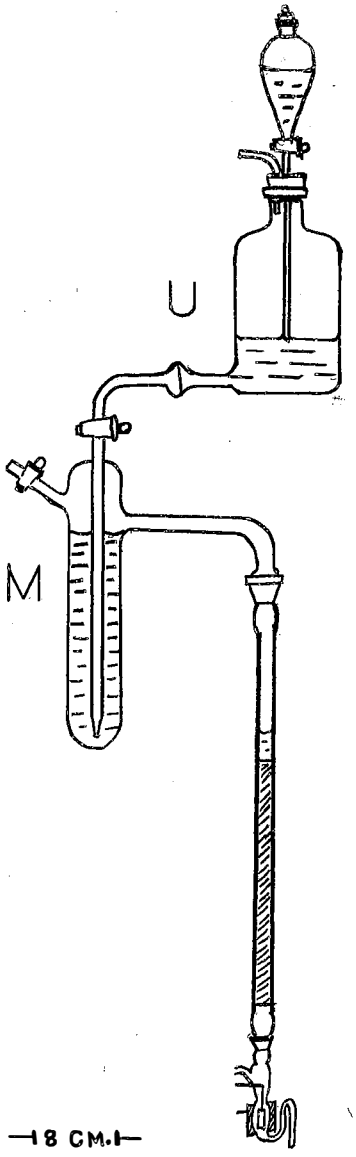

Figure 1.

Diagram of Apparatus

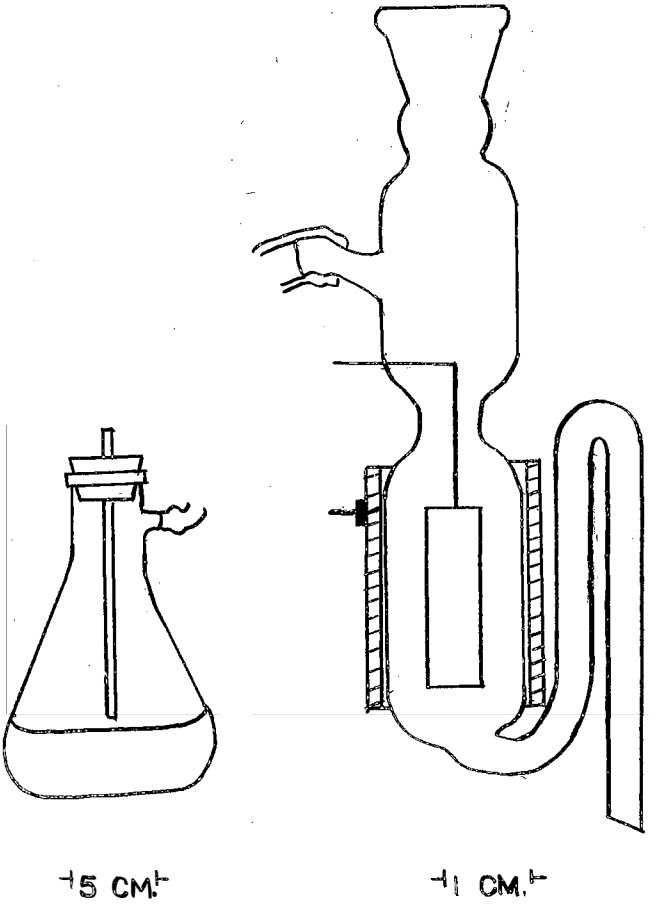

Figure 2.

Capacity Pipet-Switch

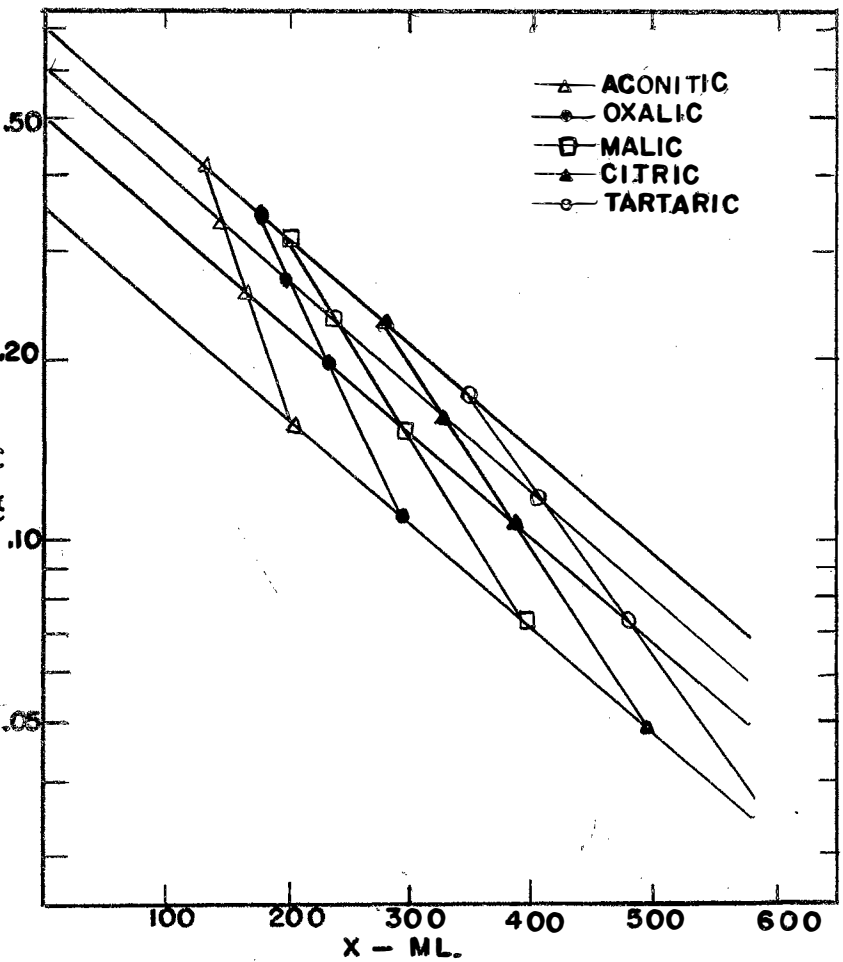

Figure 3.

Peak Volume-Concentratio

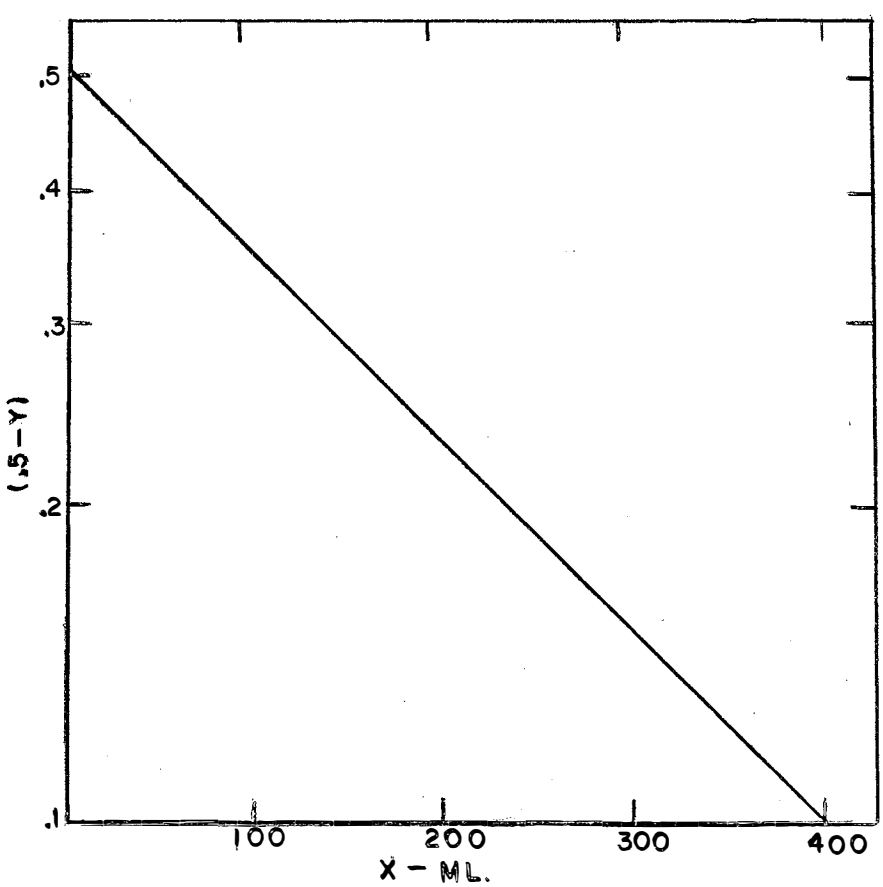

Figure 4.

Relation between Eluent Concentration and Volume

ORGANIC ACIDS OF SORGHUM SIRUP AND JUICE

REVIEW OF LITERATURE

Parsons (15) reported that the calcium salt of aconitic acid was deposited on the heating surface of evaporating pans used to concentrate sorghum juices which had previously been limed to neutrality. Wiley and Maxwell (20) working with sorghum juice considered aconitic acid to be the acid present in largest amount of those determined. They found citric, malic, acetic, tartaric, oxalic and formic acids in sorghum juice. Willaman, West and Spriestersbach (21) reported that oxalic, malic, citric, and tartaric acids were invariably present in the sorghum plant. Ventre, Ambler, Henry, Byall and Paine (18) reported aconitic acid occuring in sorghum juice both free and combined as calcium and magnesium salts. They presented an economically feasible method for extraction of aconitic acid from sorghum sirup. They reported that titratable acidity increased when the total aconitic acid content increased; however their data did not give a smooth correlation. They found that from 30 to 40 percent of the total aconitic acid could occur as free aconitic acid. Ambler, Turer and Keenan (2) identified the insoluble aconitate which separates from sorghum sirups as dicalcium magnesium aconitate hexahydrate.

SIRUP VARIETAL STUDY*

The sorghum cane used was grown on creek bottom land classified as Yahola sandy loam. Rows were spaced 42 inches apart, with the stalks averaging six inches apart. Samples representing 1/125 of an acre were secured from 100 feet of each row, thus furnishing a relatively uniform sample. The canes were stripped and headed in the field and immediately brought to a commercial mill for pressing. From the juice the sorghum sirup was prepared under laboratory conditions using the clay clarification method described by Webster, Davies and Sieglinger (19).

Sufficient sulfuric acid was added to 25 ml. of the resulting sorghum sirup to bring it to 0.5 normal when diluted to 50 ml. A 2 ml. aliquot of this solution was forced into the chromatographic column. The column was developed at a constant temperature of 25° C. A typical chromatogram is shown in Figure 7 for Collier X-8-2 sirup.

The presence of aconitic, oxalic, malic, citric, acetic, and tartaric acids was confirmed. Fumaric acid was also found to be present. The quantities of these acids found for the five varieties tested are listed in Table I. Aconitic acid was not always the predominant acid, as exemplified by Sugar Drip on an acidity equivalent basis and by Sugar Drip and Colman Y on a weight basis. In

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* Data reported in this section have been accepted for publication in Food Technology.

these two cases tartaric acid was predominant. Large varietal differences in the quantities of the acids present were found, but no general order of predominating acid existed among the varieties studied.

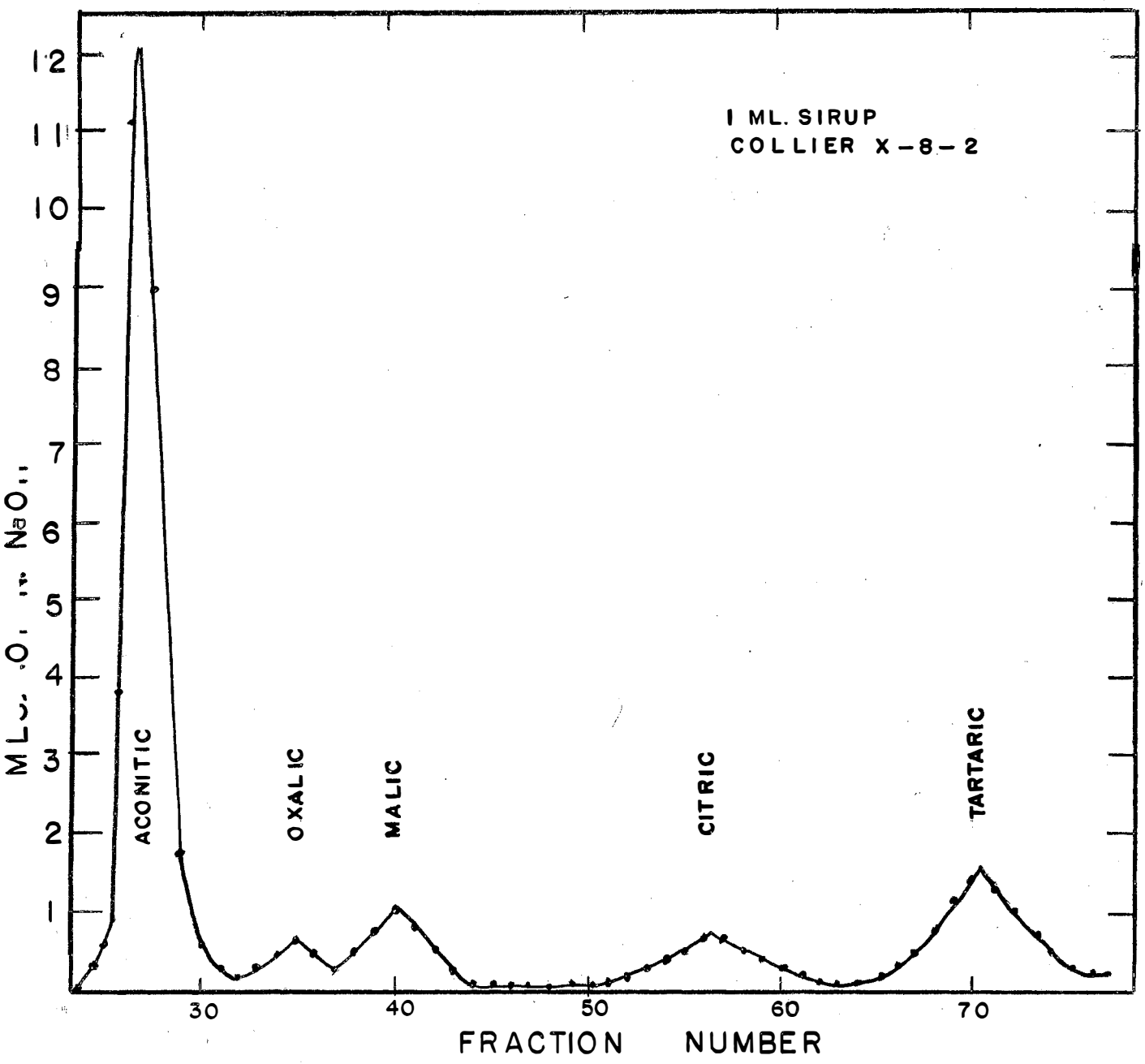

Figure 7.

A Typical Chromatogram.
System Used:
B = 250 A = .60 5.3 ml. Fractions

TABLE I

ORGANIC ACIDS OF SORGHUM SIRUP
(milliequivalents and mg. per ml. of sirup)

Variety	g. Juice per g. Sirup	Units of Acid	Aconitic	Oxalic	Malic	Citric	Tartaric	Fumaric	Acetic	Total
Lecoti	6.50	millieq./ml.	.310	.026	.070	.063	.180	.009	.004	.662
		mg./ml.	17.99	1.17	4.69	4.03	13.51	.52	.24	
Collier X-8-2	5.38	millieq./ml.	.290	.021	.036	.042	.089	.004	.002	.484
		mg./ml.	16.83	.94	2.41	2.69	6.78	.23	.12	
Colman Y	5.48	millieq./ml.	.172	.014	.036	.051	.139	.006	.004	.422
		mg./ml.	9.98	.63	2.41	3.27	10.43	.35	.24	
Sugar Drip	5.75	millieq./ml.	.084	.012	.022	.107	.174	.006	.005	.410
		mg./ml.	4.87	.54	1.48	6.85	13.06	.35	.30	
Crystal Drip	6.75	millieq./ml.	.281	.014	.059	.046	.162	.012	.008	.582
		mg./ml.	16.31	.63	3.96	2.95	12.14	.70	.48	

JUICE SEASONAL STUDY

The sorghum cane was grown on a fine sandy loam of the Yahola series, located in a bottom area of average drainage. The rows were one-quarter of a mile long, furnishing ample opportunity to secure uniform stands for sampling.

Samples were secured by stripping and heading (after the third sampling) sufficient canes to yield a gallon of juice. The juice was expressed by means of a commercial mill. The samples were taken between 8 and 9 A.M. at bi-weekly intervals.

The Leoti variety was chosen for this study because it has been reported to have high titratable acidity late in the season and has been recommended as a variety suitable for sirup manufacture (19).

The seed was planted May 11, 1953. The average height of the plants at the first sampling was 21 cm., the second sampling 44 cm., the third sampling 88 cm., and by the fourth sampling the plants had reached their maximum height of about 184 cm. During the period covered by the first four samplings the plants were in the vegetative stages and were in good condition. By the fifth sampling the seed heads were in late bloom to early milk. At the sixth sampling the seeds were in a soft to hard dough stage. At the seventh sampling the seeds were in the hard dough stage. At the eight sampling and thereafter the seeds

were ripe.

The juice was filtered and centrifuged. Two ml. of 5-normal sulfuric acid was added to 50 ml. of the juice. The resulting solution was centrifuged and its volume reduced to approximately 20 ml. by heating (35° C.) at 2-cm. pressure. The volume was further reduced to approximately 8 ml. by concentrating over phosphoric anhydride at 2-cm. pressure and 0° C. The volume was then adjusted to 10 ml. and a 2-ml. aliquot of this solution was forced into the chromatographic column. The column was developed at a constant temperature of 25° C. as previously described.

The juice was also subjected to the following determinations: density in terms of degrees Brix; sodium and potassium by the flame photometer; calcium as described in A.O.A.C. (3); magnesium using the colorimetric procedure described by Shrewsberry (17); and titratable acidity by titrating a 10-ml. sample to pH 8.0 with standard sodium hydroxide and a pH meter.

The acids found to be present were aconitic, oxalic, malic, citric, tartaric, acetic, pyruvic, fumaric, and formic. The last four are included under "Minor Acids" in Table II. The quantities of the first five acids are listed in Table II.

During the actively growing vegetative period the aconitic acid decreased to one-fifth of that present at the first sampling. During this period tartaric acid was the acid present in highest concentration, and total,

titratable, and non-titratable acidity decreased. At the late bloom to early milk stage (fifth sampling) aconitic acid began to increase and continued to do so for the remainder of the samples. At the fifth sampling the tartaric, total, and titratable acidity increased but almost no change was observed in the non-titratable acidity. By the sixth sampling time (hard to soft dough stage) and thereafter aconitic acid was the major acid, with tartaric acid decreasing. Titratable, total, and non-titratable acidity increased after the sixth sampling. These trends are shown in Figure 5 for aconitic and tartaric acids, and in Figure 6 for total, titratable, and non-titratable acidity.

Juice samples secured during September and October of 1952 were also analyzed. The trends of increasing aconitic and decreasing tartaric acid duplicated those reported in Table II for 1953.

The density in terms of degrees Brix and the cation analysis are listed in Table III. The major cation, potassium, varied randomly during the season. Calcium decreased during the first five samplings and increased thereafter. Magnesium was also at a minimum at the fourth sampling. The minimum amount of total cations occurred at the fourth sampling. The amounts of total cations did not vary over a 0.2 milliequivalent range after the third sampling. This may be a factor in the small change in non-titratable acidity over the same range.

The insoluble dicalcium magnesium aconitate hexahydrate reported by Ambler, Turer and Keenan (2) could account for precipitation from sirup of half of the aconitic acid present during the ripe stages if the calcium is considered as the limiting factor. Therefore, as reported by Ventre, Ambler, Henry, Byall and Paine (18), considerably more calcium must be added to the sirup to precipitate all the aconitic acid, and in excess of that required for neutralization of the free acids, since considerable aconitic acid will be in the form of the soluble potassium salt.

The aconitic acid fractions from five separations were combined and extracted with ether. The ether was evaporated and the resulting aconitic acid crystals were recrystallized from water-free alcohol. The aconitic acid melted at 190° C. Since the melting point of cis-aconitic acid is 125° C. and that of trans-aconitic acid is 190-197° C., the acid separated was the trans form. Owing to the unstable nature of the cis-aconitic acid isomer it is possible that any originally present was converted to the trans form during the preparation of the sample.

TABLE II

VARIATION OF ORGANIC ACIDS AND THEIR ANIONS
IN SORGHUM JUICE AT VARIOUS STAGES OF MATURITY
(millieq./10 ml. juice)

Sampling No.	Sampling Date	Density Brix°	Acid							Titratable Acidity	Non-Titratable Acidity
			Aconitic	Oxalic	Malic	Citric	Tartaric	Minor	Total		
1	6-15-53	4.7	.560	.010	.020	.097	.548	.019	1.254	.296	.958
2	6-29-53	4.6	.315	.017	.014	.066	.584	.013	1.009	.220	.789
3	7-13-53	4.5	.233	.005	.007	.068	.428	.007	.748	.077	.671
4	7-27-53	6.0	.113	.007	.005	.146	.380	.012	.663	.074	.589
5	8-10-53	11.4	.165	.014	.024	.070	.520	.014	.807	.209	.598
6	8-24-53	14.8	.416	.010	.014	.069	.277	.012	.798	.174	.624
7	9-7-53	15.4	.577	.009	.016	.068	.251	.011	.932	.230	.702
8	9-21-53	17.3	.784	.018	.024	.086	.131	.010	1.053	.360	.693
9	10-5-53	17.6	.855	.010	.022	.101	.126	.014	1.128	.373	.755
10	10-19-53	17.6	.890	.025	.033	.068	.119	.015	1.150	.419	.731

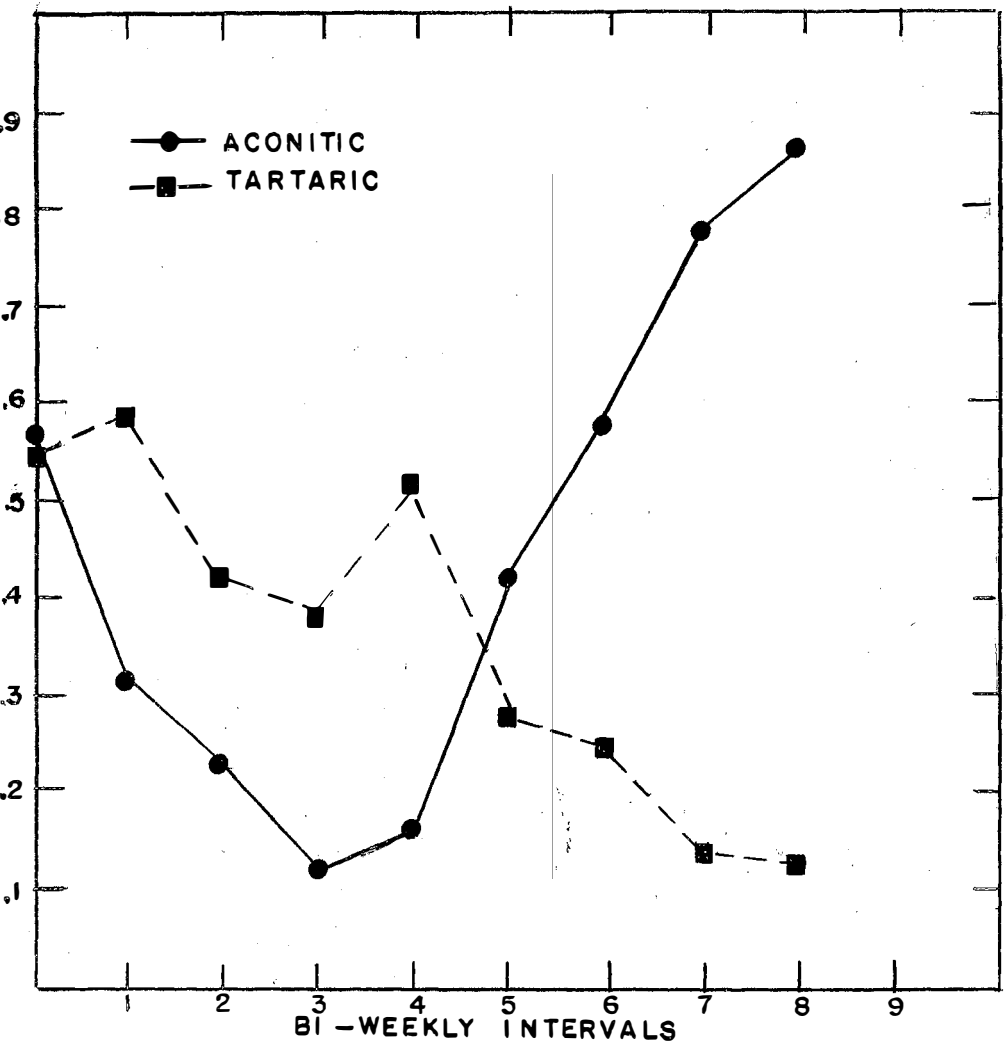

Figure 5.

Seasonal Variation o
Aconitic and Tartari
Acids of Sorghum Juic

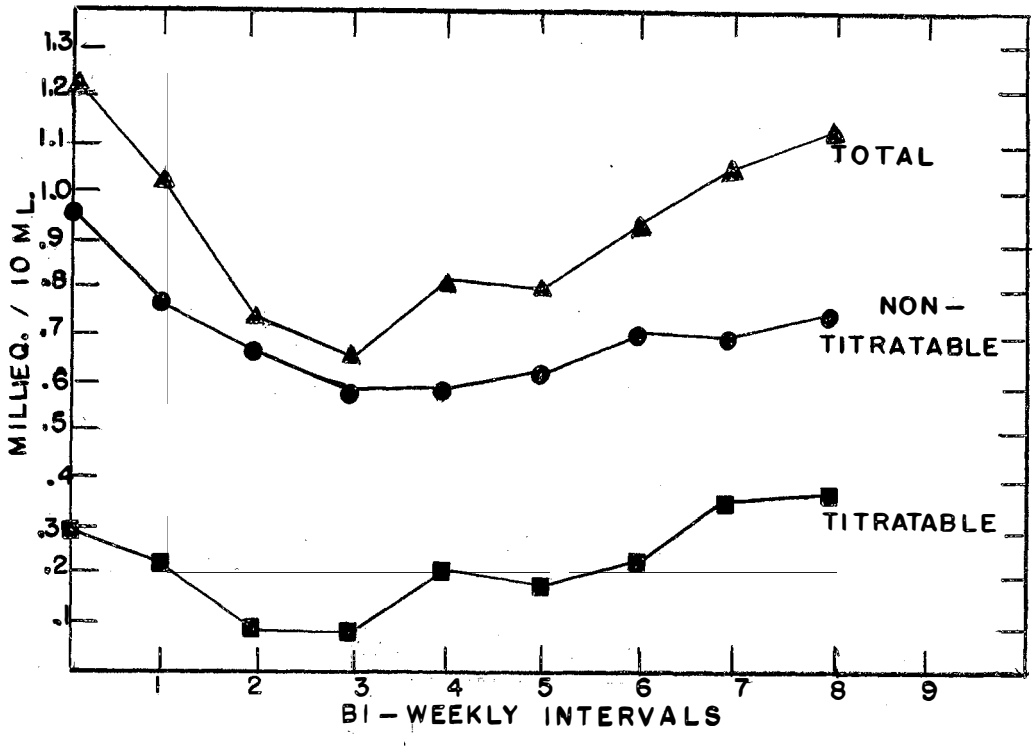

Figure 6.

asonal Variation of
tal, Titratable and
n-Titratable Acids
Sorghum Juice

TABLE III

DENSITY AND CATION CONTENT OF SORGHUM JUICE
(millieq./10 ml. juice)

ampling No.	Density, Brix°	Potassium	Magnesium	Calcium	Sodium	Total Cations
1	4.7	.782	.346	.270	.019	1.417
2	4.6	1.000	.354	.255	.019	1.628
3	4.5	.480	.387	.250	.037	1.154
4	6.0	.525	.229	.240	.011	1.005
5	11.4	.703	.158	.170	.007	1.038
6	14.8	.828	.162	.200	.006	1.196
7	15.4	.767	.183	.225	.020	1.195
8	17.3	.734	.204	.265	.005	1.208
9	17.6	.595	.179	.315	.005	1.094

SUMMARY

Aconitic acid is not always the predominant acid of sorghum sirup. The varieties Sugar Drip on an acidity equivalent basis and Sugar Drip and Colman Y on a weight basis contained chiefly tartaric acid. Large varietal differences in the quantities of the acids present were found.

Tartaric acid has been found to be the major acid of Leoti sorghum juice until after the hard dough stage. During the vegetative period aconitic acid decreased. At about the time of the hard dough stage the aconitic acid increased to predominance and continued to increase thereafter with tartaric acid decreasing. The increasing titratable acidity found late in the season (18) is hence a result of the rapid increase in aconitic acid.

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II. ORGANIC ACIDS OF SORGHUM SIRUP AND JUICE

AUTHOR: Charles Lavern Mader

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